

Determining Baseline Stress-Related Hormone Values in Large Cetaceans

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LONG-TERM GOALS

The long-term goals of this project are four-fold: 1) determine stress-related hormone (cortisol) baseline values from reconstructed lifetime hormone and contaminant profiles in both historic (<1970s) and contemporary archived whale earplugs (>1980s), 2) determine the potential relationship between stress-related hormones and contaminants concentrations within an individual earplug, 3) compare and contrast stress-related hormones and contaminants levels between historical and contemporary earplug samples (among species), and 4) determine the potential relationship between stress-related hormones recovered in earplugs with hormones recovered from blubber. From these data, we will ***establish species specific baseline levels that can be used as a comparative tool in future cetacean stress research***. The results of this study will contribute to improving mitigation strategies through improved assessments of the potential impacts of anthropogenic activity.

OBJECTIVES

Historically, whale earplugs have been used to age large cetaceans. This rudimentary technique which consists of vertically slicing the earplug and counting the light and dark lamina (Figure 1 and 2) is similar to tree ring-dating techniques. We are now able to combine historical uses (aging techniques) with a unique analytical approach to reconstruct chemical profiles (chronology) from an individual whale. ***These reconstructed chemical profiles provided a unique window into stress-related hormone (cortisol, aldosterone, T_3 and T_4) concentrations and variability during periods of stress, such as development and contaminant exposure.*** As such, whale earplugs are capable of providing an invaluable record or profile of stress levels and environmental exposure to contaminants that is not available using any other technique. By analyzing the large collection of historical earplugs that are currently archived in museums and to which we have been granted access (Table 1), we will assess baseline stress and the relationship between stress and exposure levels of baleen whales. This approach to examine lifetime stress-related hormonal levels will be transformative for our understanding of the extent of exposure and the potential effect on the health of these animals and offers exponential research opportunities that simply does not exist with traditional matrices such as blood and blubber and is ideally suited for examining stress-related hormones over an organism's lifetime.

1.1 Objectives

1. Determine stress-related hormone baseline values from reconstructed lifetime hormone and contaminant profiles in both historic (<1970s) and contemporary archived whale earplugs (>1980s).
2. Determine the potential relationship between stress-related hormones and contaminants concentrations within an individual organism (earplug).
3. Compare and contrast stress-related hormones and contaminants levels between historical and contemporary earplug samples (among species).
4. Determine the potential relationship between stress-related hormones recovered in earplugs with hormones recovered from blubber.

Specific Objectives:

Objective 1:

Determine stress-related hormone baseline values from reconstructed lifetime hormone and contaminant profiles in both historic (<1980s) and contemporary archived whale earplugs (>1980s). To meet this objective, we will utilize research collaborations developed with some of the world's most prestigious museums, such as the Smithsonian Institution in Washington DC and the London Museum of Natural History. The PIs currently have the necessary NMFS permit and access to approximately 150 identified individual earplugs from different whale species such as blue whales, fin whales, grey whales, minke whales, bowhead whales and humpback whales (permits, letters of support, and Table 1). It should be noted that the PIs have been in direct and constant communication with the Smithsonian Institution (Museum of Natural History; Charles Potter) regarding its 450 cetacean earplugs specifically identified (with other tissues included) to be used for this proposed research. We will utilize ultra-trace analytical techniques recently developed at Baylor University, which are capable of measuring stress-related hormones and anthropogenic contaminants in 0.1 – 0.5 g cerumen samples. These data will also ***provide the first estimates in lifetime variability (both spatially and temporally) in stress-related hormone and contaminant levels in cetaceans.***

Objective 2:

Determine the relationship between stress-related hormones and contaminants concentrations within an individual organism (earplug).

We will assess the statistical relationship and develop a model between stress-related hormones and contaminant levels for an individual organism over the organism's entire lifespan (i.e. within an earplug). Because contaminants are a known stressor for mammals, understanding this relationship is a key first step in providing the degree in which these variables initiate a known stress response.

Objective 3:

Compare and contrast stress-related hormones and contaminants levels between historical and contemporary earplug samples (among species).

We will determine stress-related hormones differences and variations as well as temporal differences associated with archived historical and contemporary earplugs. Stress-related hormones as well as contaminants will be assessed as lifetime profiles (as a percent change from baseline; see preliminary results, Figure 3) in historical and contemporary earplugs and differences assessed. For example, we expect natural variation or percent change from baseline concentrations in all historic and contemporary samples; however, we predict that those species subjected to a higher degree of stress

(i.e. anthropogenic contaminants or contemporary events such as chronic sound) than natural levels will show a greater degree of variation and percent change from baseline values. This will provide the first data on variations among individuals and species in stress-related hormones as well as temporal differences in whale stress levels. Note: Due to the half-life of cortisol as well as the time resolution of earplugs, we predict no spike in cerumen (wax) stress-related hormone levels associated with capture methods used prior to the Marine Mammal Protection Act of 1972. In other words, there will be no stress marker associated with a short-term or acute stressor such as a capture episode.

Objective 4:

Determine relationship between stress-related hormones recovered in earplugs with hormones recovered from blubber.

We will determine the relationship between stress-related hormones recovered from cerumen as well as blubber from the same animal. While blubber cortisol concentrations will be a reflection of an indistinguishable time frame from recent exposure, our analysis will provide correction factors from the cerumen of the last secreted lamina of the earplug with that of the blubber. In our previous research, the lifetime accumulative contaminants burden was recorded in the lamina of the earplug of a male blue whale and totaled $5,200 \text{ ng} \cdot \text{g}^{-1}$. Blubber tissue collected from the same whale provided a total contaminant burden of $4,700 \text{ ng} \cdot \text{g}^{-1}$ lipid or 90% of the total accumulative burden recorded in the earplug. This 10% decrease may be due to differences in uptake, metabolism, and excretion process specific to blubber compared with cerumen. Specifically, *o,p'*-DDE, *p,p'*-DDE, *p,p'*-DDT, *cis*- and *trans*-nonachlor, PCB 105, 118, 138, 153, 156, and 187, as well as PBDE 47, 99, and 100 were identified in both blue whale earplug cerumen and blubber. The major difference between the two matrices is that blubber cannot estimate when the exposure transpired, whereas earplugs provide lifetime accumulative contaminant burden with, in this case, a 6-mo resolution. Understanding the relationship between the earplug (sequential stress profile) with blubber (non-sequential stress profile) will provide 1) extent of differences between matrices and 2) a model of how stress-related hormones are represented relative to the matrix and temporal nature of the stressor. These species-specific correction factors will be invaluable in future stress research utilizing blubber sampling.

APPROACH

Sectioning whale earplugs

Aliquots of each layer of the frozen whale earplug will be sectioned using a high-speed drill (Dremel 4000 High Performance Rotary Tool, Racine, WI). Earplugs will be sectioned longitudinally up to four times, depending on its thickness to improve accessibility of the internal lamina using an ultra fine-toothed band saw (Vectrax Vertical Variable Speed Band Saw, MSC Industrial Supply Co., Melville, NY) operating from 350–1200 rpm. The earplug will be manually moved through the band saw at approximately 0.5–1 fpm. Serial sections will be labeled and stored at -80°C . For lamina discrimination (aging), each section will be photographed using a high-resolution digital camera (12MP) and photographic software (Canon U.S.A.[®]). Under 20X magnification, each lamina layer will be removed from each longitudinal section (Figure 2). Lamina aliquots will be placed into vials with PTFE caps and stored under nitrogen at -30°C .

Stress-related hormone radioimmunoassay technique

Cortisol, aldosterone, hormones thyroxine (T_4) and triiodothyronine (T_3) levels in each identified lamina will be determined utilizing Correlate-EIA kits (ENZO Assay Design). Aliquots of cerumen/lamina will be homogenized and transferred to a (2:1) chloroform:methanol solution where lipids will be extracted for 60 min at 160°C in a Soxtec 2043 (Foss[®], Eden Prairie, MN) and

subsequently pipetted into polypropylene tubes coated with antibodies against the hormones tested. One milliliter of radio-labeled hormone will be added and incubated at 37 °C for 45 min before decantation. The amount of antibody-bound labeled hormone will be assessed against standard calibration curves.

For example, for cortisol calibration curve (with six points ranging from 0.24 to 7.8 pg mL⁻¹) and check curve, a sample containing > 7.8 pg mL⁻¹ cortisol will be diluted 5 times 1:2 in the kit assay buffer and measured in the assay. These data will be plotted graphically as actual cortisol concentration versus measured cortisol concentration. Intra-assay precision will be determined by taking samples containing 156 pg mL⁻¹, 1250 pg mL⁻¹ and 5000 pg mL⁻¹ concentrations of cortisol and running these samples multiple times in the same assay. Sample recoveries for cortisol will be measured using duplicate cerumen aliquots.

Contaminant extraction methodology

POPs will be extracted from cerumen homogenates using selective pressurized liquid extraction (SPLE), which combines pressurized liquid extraction (PLE) with adsorbent cleanup techniques into a single automated technique (Robinson et al. *in revision*; Figure 5). SPLE will be performed using an accelerated solvent extractor (ASE 350, Dionex, Salt Lake City, UT) with 66 mL ASE extraction cells. The final method will consist of homogenization of an aliquot of whale cerumen (~0.25 g) with sodium sulfate (baked at 500 °C for 12 hours and allowed to cool) utilizing a mortar and pestle. Cerumen homogenates will be placed on top of pre-cleaned sorbents (with an order of basic alumina oxide, silica gel and florisil from top to bottom) within the ASE cell (Figure 5). The sorbents will be pre-cleaned using 1:1 (v/v) dichloromethane (DCM):hexane (HEX) under the following ASE conditions: 100 °C, 1500 psi, and 50% rinse volume. Cerumen homogenates will be spiked with isotopically-labeled surrogate standards to correct for target analyte loss during sample preparation and will be allowed to come to equilibrium for 1 hr prior to extraction. Next, cerumen homogenates will be extracted with DCM:HEX (1:1) under the same ASE conditions as described above except with a 150% rinse volume. ASE extracts will be concentrated to ~0.3 mL using a Turbo Vap II from Caliper (Hopkinton, MA), then transferred to a gas chromatography (GC) vial and spiked with isotopically-labeled internal standards prior to analysis.

Contaminant Extract Analyses

The analysis of pesticides, PCBs, and PBDEs will be performed using a 7890 gas chromatograph coupled to a 5975 mass spectrometer (Agilent Technologies, Santa Clara, CA) in electron capture negative ionization (ECNI) or electron impact (EI) with selective ion monitoring. One microliter of sample extract will be injected utilizing an Agilent 7683 Injector in a pulsed splitless mode (pulse at 20 psi until 0.74 minutes). The injection port will be set to 300 °C. Chromatographic separation will be achieved using a DB-5 capillary column (J&W, 30 m x 0.25 mm i.d.; 0.25 μm film thickness). All analytes except for *p,p'*-DDT, *p,p'*-DDE, and *o,p'*-DDE will use an oven temperature program of 120 °C, held for 1 min, ramped to 275 °C at 4 °C min⁻¹, then ramped to 320 °C at 6 °C min⁻¹, and held for the final 5 minutes. The total run time will be 52.25 minutes. Helium (99.999%) will be used as the carrier gas, and methane (99.999%) will be the buffer gas. The ECNI ion source and quadrupole mass analyzer temperatures will both be set to 150 °C.

p,p'-DDT, *p,p'*-DDE, and *o,p'*-DDE will be analyzed using the same instrumentation and parameters as described above except in EI mode with an oven temperature program of 120 °C for 1 minute, and then ramped at 4 °C min⁻¹ to 250 °C for a total run time of 33.5 minutes and a source temperature of 230 °C. The quadrupole mass analyzer temperature will be 150 °C.

Quality Assurance and Quality Control (QA/QC)

Target analytes will be identified based on retention times (± 0.05 min) as well as a quantitative to qualitative ion response ratio ($\pm 20\%$). Ion responses ratios will be based on continuous calibration verification standards analyzed prior to sample analysis. All target analytes will be identified using a single quantitative ion and two qualitative ions, except for *p,p'*- DDT, which has one quantitative ion and one qualitative ion. Following these QA/QC guidelines, a representative chromatogram showing the first identification of trans-chlordane and trans-nonachlor in cerumen is provide in Figure 6. Target analyte concentrations will be determined using a calibration curve with at least seven points ranging several orders of magnitude. Target analyte calibration curves will plot the response dependent concentration factor of the target analyte (concentration of target analyte divided by the concentration of its surrogate standard) versus the concentration dependent response factor of the surrogate standard (response of the target analyte divided by the response of its surrogate standard). Target analyte calibration curves will be linear and forced through the origin, and have coefficients of determination (r^2) of at least 0.99. Surrogate recoveries will be quantified using internal standards spike prior to analysis.

Calibration curve check standards will be run before and after each sample batch to validate the integrity of the calibration curve. Calibration curves and calibration check standards will be prepared simultaneously. Calibration check standards concentrations will correspond to the upper-middle point of the calibration curve. Calibration check standard concentrations not within $\pm 30\%$ of the prepared concentration will require instrument maintenance and subsequent re-running of the sample batch.

Based upon the aforementioned analytical method and QA/QC protocols, a triplicate spike and recovery experiment using blue whale cerumen has been performed. The triplicate average recoveries for organochlorine pesticides, polychlorinated biphenyls, and polybrominated diphenyl ethers were 91, 93, and 76%, respectively (Robinson et al. *in revision*; Table 2). These experiments served to validate this analytical method for identification and quantification of POPs in cerumen.

Technical Approach

Objective 2:

Regression techniques (linear, curve fitting) will be used to assess the relationships among age (based on age/lamina counts), hormone, and contaminant concentrations (lipid mass basis) among species of large whales sampled. While estimating concentrations based on lipid mass will account for foraging changes, we will also eliminate the effect of body size in this comparative analysis. A majority of the earplugs from museums holdings have associated lengths at time of sampling. We will back calculate age at length regression using published length-mass regressions ($\log_e M_{mn} = a + b \log_e L_{max}$) and estimate growth curves and, hence, eliminate the effect of body size in our hormone and contaminant analyses. For example, published blue whale regressions (male, $r^2 = 0.97$, $n = 6$; $a = -7.347$, $b = 2.329$) were used to determine mass at age (lamina counts) (Trites and Pauly 1998).

Technical Approach

Objective 3:

Baseline hormone concentration will be determined within a specific earplug (see Figure 3). To determine significant changes in hormone levels from baseline, a Wilcoxon signed-rank tests will be performed. Based on previous results, we anticipate a positive skew of the hormone distributions, therefore values will be log transformed. Correlations between age, hormone and contaminant concentrations will be calculated using Pearson correlation coefficients. These measurements will include all hormones (aldosterone, T_3 , T_4 , and cortisol) as well as contaminants. The age trends for the

hormones will be calculated by subtracting baseline log hormone levels from the log hormone levels for each individual and then dividing the result by the amount of time between the two measurements (expected 6 to 12 months). This yields the rate of change per year for each individual on the log scale. The sample mean and the standard error of the rates will be calculated. Percentage change will be estimated and transformed back to the original scale by substituting the sample mean in for “b” in the formula such that the percent change per is:

$$\text{Year} = 100\% \times (e^b - 1)$$

The standard error of the percent change will be computed by substituting the standard error in for “b” in this formula.

Technical Approach

Objective 4:

Stress-related hormone (cortisol, aldosterone, T₃ and T₄) levels in the earplug and corresponding blubber samples will be determined by radioimmunoassay techniques (see above). Correction factors will be determined for each hormone for each sample set analyzed. Mean values and therefore mean correction factors will be assigned to each species.

WORK COMPLETED

This award was announced in June 2014; thus far progress has been made in securing archived samples from the Smithsonian Museum as well as the London Museum of Natural History. Thus far we have collected a total of 26 archived earplugs from museum holdings from 5 species; blue (n=7), fin (n=14), grey (n=1), minke (n=1) and humpback whales (n=2) as well as 3 freshly collected earplugs from strandings/harvest (bowhead (n=1), fin (n=1) and blue (n=1), N=29. We are also working with Dr. Alex Aquilar in Barcelona Spain to gain access to his collection of 300+ earplugs collected during the 1970s and 1980s. Our team has also been in contact with stranding networks along both U.S. coasts as well as in Spain to be notified in the event of a baleen whale stranding. Our team has added one post-doctoral colleague to this project to begin to develop models using these data. We are in the mid-phases of this study and have analyzed one blue whale earplug for cortisol and persistent organic contaminants as well as hormone profiles from one female bowhead earplug collected in 2013 (>1980s) from Barrow Alaska. The following table consists of the samples collected thus far from both museum holdings as well as fresh carcass samples. It should be noted that as part of our collection we have secured the oldest earplug (c. 1909) known in the world. This earplug was part of the collection of D.G. Lillie (1910) and his sentinel work describing the earplug and how it could be used as an aging tool. This earplug is estimated to be 35 years old (1874-1909) and will yield stress data (baseline) prior to ship noise, contaminants or other modern anthropogenic stressors. The plugs collected from both archived and fresh samples represent 6 species from the Pacific, Atlantic, Southern Oceans as well as the Bering Sea.

Table 1. Balaenopteridae earplugs collected for ONR project along with sex, where samples originated, estimated age and date collected. Asterix* represents an estimation of age based on initial count of growth layers. Some unknowns (#) such as sex will be determined by hormone analysis. The bold highlighted earplug is the oldest known sample in the world.

	Species	Sex	Region of sampling	Estimated age*	Date collected
1	Balaenoptera musculus	M	Pacific Coast	12	2007
2	Balaenoptera musculus	#	Southern Ocean	36	1956
3	Balaenoptera musculus	#	Southern Ocean	41	1956
4	Balaenoptera musculus	F	Southern Ocean	50+	1955
5	Balaenoptera musculus	F	Southern Ocean	50+	1955
6	Balaenoptera musculus	M	Southern Ocean	35	1956
7	Balaenoptera musculus	F	North Pacific	33	1964
8	Balaenoptera musculus	M	North Pacific	30	1965
9	Balaenoptera physalus	#	North Atlantic	35	1909
10	Balaenoptera physalus	F	North Atlantic	42	1963
11	Balaenoptera physalus	M	North Atlantic	29	1956
12	Balaenoptera physalus	F	North Atlantic	14	1956
13	Balaenoptera physalus	M	North Atlantic	20	1956
14	Balaenoptera physalus	#	North Atlantic	18	1955
15	Balaenoptera physalus	M	North Atlantic	36	1955
16	Balaenoptera physalus	M	North Atlantic	28	1955
17	Balaenoptera physalus	M	North Atlantic	31	1955
18	Balaenoptera physalus	#	North Atlantic	32	1930
19	Balaenoptera physalus	#	Southern Ocean	30	1955-56
20	Balaenoptera physalus	M	Pacific Coast	7	1964
21	Balaenoptera physalus	F	Pacific Coast	17	1961
22	Balaenoptera physalus	M	Pacific Coast	2-3	2015
23	Balaenoptera physalus	F	Pacific Coast	24	1967
24	Balaenoptera acutorostrata	M	North Pacific	86	1964
25	Megaptera novaeangliae	M	Pacific Coast	58	1961
26	Megaptera novaeangliae	F	Pacific Coast	38	1961
27	Eschrichtius robustus	F	Pacific Coast	27	1967
28	Eschrichtius robustus	M	Pacific Coast	7	2011
29	Balaena mysticetus	F	Bering Sea	65	2013

Age estimations were determined for each earplug based on the counting of the lamina (light and dark lamina). Each growth layer (1LL + 1DL) follows previous age estimation studies in which it was determined that each GL equalled one year. The total collection of earplugs from all species can be used to reconstruct chemical profiles of whales spanning 1874-2013. We have 2 animals that will provide chemical profiles of whales from the 19th century.

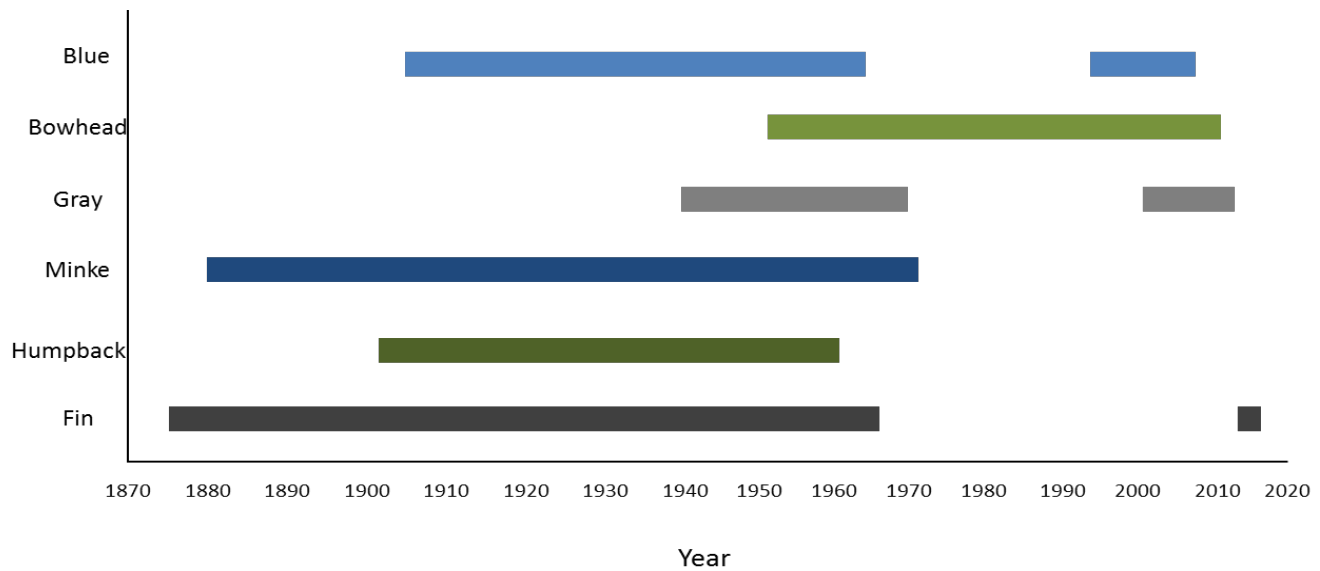


Figure 1. Span of years for chemical analysis from whale earplugs collected from archival and fresh samples.

RESULTS

Blue whale

One blue whale earplug has been analyzed for contaminants and the stress hormone cortisol (N=1; 12 growth layers (GL)). This earplug was extracted from a shipstrike animal in 2007 (>1980s) and housed at the Santa Barbara Museum of Natural History until shipped to Baylor. Aging analysis produced an estimation of 144 months (± 6 months; dark and light lamina = 1 year) using high resolution digital imaging. A baseline cortisol was established at approximately 12 months of age (red dot). While cortisol levels increased over the lifespan of this animal (Figure 2), two distinct increases, possibly corresponding with development (birth to juvenile and juvenile to sexual maturation), were detected using percent change over baseline cortisol concentrations.

Bowhead Whale

One earplug from a bowhead whale was acquired from Barrow Alaska collected from subsistence harvests in the summer of 2013 (B1-2013; 53 cm, 800g). Sixty-five (65) distinct GLs have been identified from this bowhead whale (light layer = 52% lipid; dark layer = 25% lipid; Figure 3). This corresponds to a time frame of 1949-2013 for all reconstructed chemical profiles. Thus far we have identified three hormones; two pregnancy (estradiol and progesterone) and cortisol for stress. Figure 3 reveals several distinct hormone peaks which most likely correspond to pregnancy episodes and the possible stress response of the female. However, a very large single cortisol peak is evident during the mid-late 1960s. This may be in response to first mating or possible anthropogenic events occurring in the Bering-Chukchi Sea waters. This single cortisol peak effects the overall mean cortisol levels such that pre 1980 levels are significantly higher than post 1980 levels even though levels are more constantly higher with greater variability post 1980 (t-test $p < 0.05$; Figure 4).

Over the next year all collected samples will be analyzed for a series of hormones and contaminants. Also samples from archived collections will be accessed as well as increasing sample size of recent samples from stranded dead carcasses.

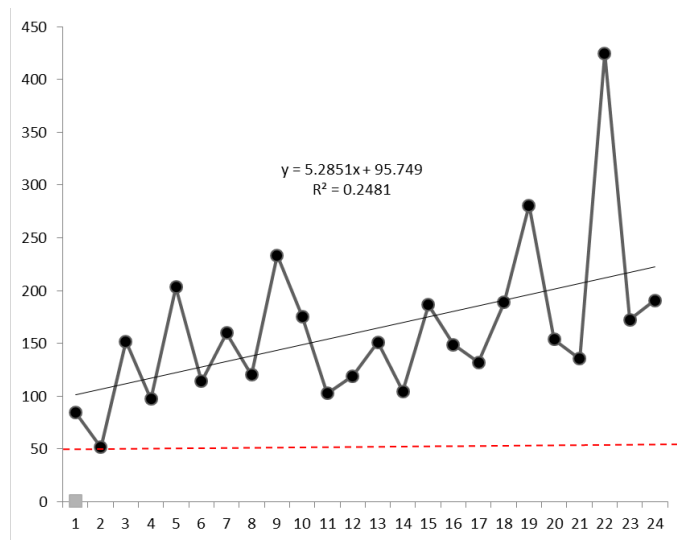


Figure 2. Cortisol levels reconstructed from an earplug collected from a 12 yo (12 GL) blue whale male. The red line corresponds to the baseline cortisol levels detected.

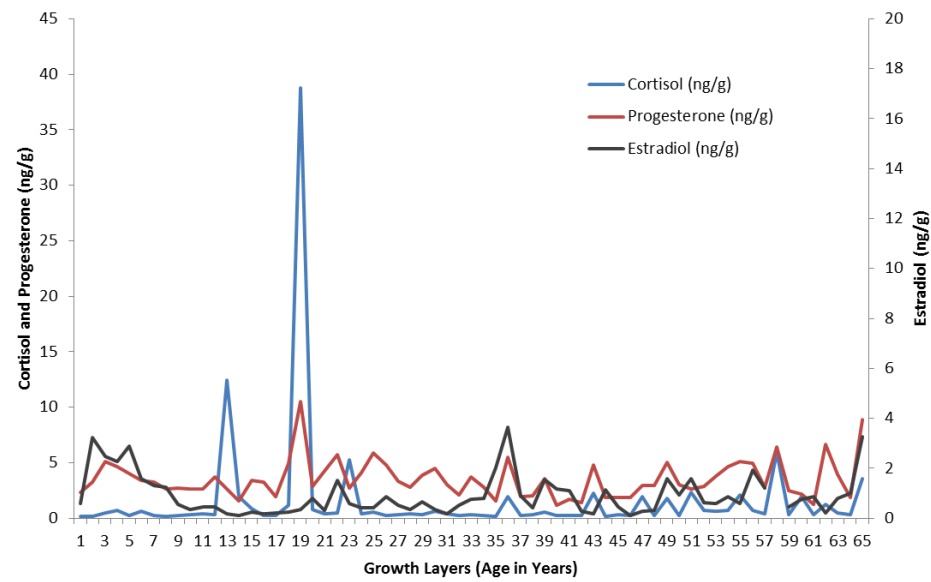


Figure 3. Hormone reconstruction including cortisol, progesterone and estradiol from an earplug of a 65 (GL) bowhead whale.

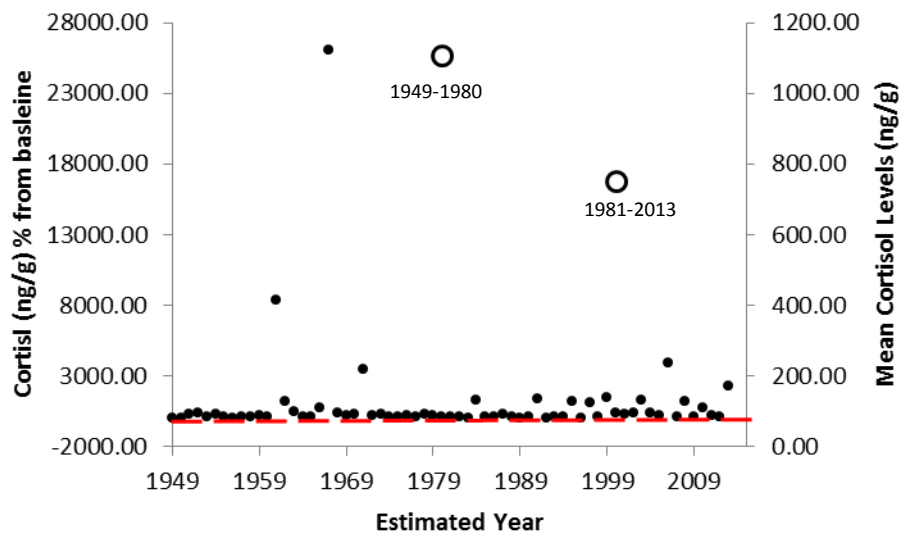


Figure 4. Cortisol levels reconstructed from the earplug of a 65 yo (GL) female bowhead whale. Baseline is red line. Open circles represent mean values pre and post 1980.

IMPACT/APPLICATIONS

The Office of Naval Research's Marine Mammal Physiology Program (Code 32) seeks to develop an understanding of the natural variation of stress markers; better understand and characterize the relationships among hormones or other biomarkers in different matrices; define and compare the quantitative and temporal relationships of hormones across the different matrices; and evaluate/characterize the relationship between the physiological stress response in marine mammals and acoustic exposure and 'biologically significant' disturbances. This study is aimed at determining baseline stress-hormone concentrations as well as the influence of various anthropogenic influences (contaminants) on the stress response of large whales (mysticetes). Current methods associated with analyzing the effects of anthropogenic activities on the marine ecosystem are labor, time, and cost intensive while offering only a recent snapshot of an event. As a result, there are no feasible approaches to assess baseline stress-related hormones in marine mammals. Without such data, there is no context with which to interpret the biological significance or anthropogenic impact on individuals and populations. The proposed study will report baseline stress-related hormone levels from the whale earplugs archived in museums and offer a correction factor with those hormone levels taken from blubber. From these data, we will ***establish species specific baseline levels that can be used as a comparative tool in future cetacean stress research.*** The results of this study will contribute to improving mitigation strategies through improved assessments of the potential impacts of anthropogenic activity.

Because we have access to samples dating back to the 19th century, lifetime profiles of these analytes for several species of large whales will provide, for the first time ever, a clear picture of the impact of anthropogenic disturbances (e.g. noise disturbance). We believe that this will assist the Navy in explaining the impact, or lack thereof, in marine mammals and stress associated with noise (low frequency). Our study will also improve our understanding of the natural physiological responses associated with development in marine mammals.

RELATED PROJECTS

There are no related products

REFERENCES

Lillie, D. G. 1910. Observations on the anatomy and general biology of some members of the larger Cetacea. Proc. zool. Soc. Lond. (1910), 769-92.